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Isolation and Partial Characterization of the Pink and Blue Pigments of Pocilloporid and Acroporid Corals

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Abstract. The compounds responsible for the pink and blue colors of two families of hermatypic corals (Pocilloporidae, Acroporidae) from the southern Great Barrier Reef were isolated and biochemically characterized. Isolation of the pink pigment from *Pocillopora damicornis* (named pocilloporin, λ_{max} = 560 nm, 390 nm) revealed that it was a hydrophilic protein dimer with a native molecular weight of approximately 54 kD and subunits of 28 kD. The subunits are not linked by disulfide bonds. Attempts to dissociate the chromophore from the protein proved unsuccessful. Denaturing the protein with heat (60°C) or 5% sodium dodecyl sulfate (SDS) removed the 560-nm absorbance peak without introducing a detectable bathochromic shift. In acetone, ethanol, ether, and chloroform, the pigment precipitates out of solution, leaving a colorless supernatant. These properties suggest that the protein and chromophore are covalently linked. Ion analysis revealed that the pigment does not have metal ions chelated to it. Coral pigments were also isolated from pink morphs of other pocilloporids, *Seriatopora hystrix* (λ_{max} = 560 nm) and *Stylophora pistillata* (λ_{max} = 560 nm); and from bluish regions of the acroporids, *Acropora formosa* (blue; λ_{max} = 590 nm) and *Acropora digitifera* (purple; λ_{max} = 580 nm). With the exception of *A. formosa*, all the corals examined had pigments with the same native (54 kD) and subunit (28 kD) molecular weights as those of *P. damicornis*. *A. formosa* pigment has a native molecular weight of about 82.6 kD and three subunits of 28 kD. The pigments isolated from each of these coral species have properties similar to those described for *P. damicornis*. Isolation and biochemical purification of the pigment enabled the exploration of the function of the pink pigment. Three possibilities were eliminated. The compound does not act as (i) a photoprotectant for shielding the photosynthetic pigments of symbiotic zooxanthellae against excessive irradiances, (ii) a fluorescent coupling agent for amplifying the levels of photosynthetically active radiation available for resident zooxanthellae, or (iii) a UV-screen against the high UV levels of shallow tropical marine environments.

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Introduction

The vivid colors of reef-building corals and other invertebrates are among the most conspicuous elements of a living coral reef. With this in mind, it is perhaps surprising that so little is known about the identity and role of color in reef-associated organisms (Czeczuga, 1983). The pigmentation of reef-building corals occurs in the skeleton of some species and in the ectodermal and endodermal tissues of others (Kawaguti, 1944; Takabayashi and Hoegh-Guldberg, 1995). The chemical identities of compounds responsible for coral color are known only in a few cases. Pigments associated with some hydrocoral and scleractinian coral skeletons have been identified as carotenoprotein complexes (Fox and Wilkie, 1970; Fox, 1972; Ronneberg *et al.*, 1979). Red and green carotenoprotein complexes are also present in askeletal cnidarians such as *Actinia equina* and *Epiactis prolifera* (Czeczuga, 1983). The blue pigment from the skeleton of the hydrocoral *Heliopora caerulea* has been identified as a calcium-bonded biliverdin, which belongs to the tetrapyrrole group of pigments (Tixier, 1945, cited in Fox and Wilkie, 1970). Among the most prominent pigments associated with the tissues of corals are the pinky-mauve pigments that are typical of the Pocilloporidae, Acroporidae, Poritidae, Fungiidae, and Meruliniidae (5 out of the 16 families of reef-building corals, Veron, 1986). Although several skeletal pigments have been purified and identified, the nature of these tissue-associated pigments in corals remains unexplored.

Tissue-based pigments have been extracted from corals in early studies using distilled water or buffer solutions

(Kawaguti, 1944; Shibata, 1969), but further purification and characterization have not been attempted. The Indo-Pacific coral *Pocillopora damicornis* from One Tree Island (southern Great Barrier Reef) shows a range of colony colors from pink to brown. In this case, the color has been identified as due to a hydrophilic compound found in the cells of the coral; the compound is inducible by visible light in the pink morph of *P. damicornis* (Takabayashi and Hoegh-Guldberg, 1995). Interestingly, the presence of this compound in pink morphs is associated with several physiological characteristics such as reduced growth rates (Takabayashi and Hoegh-Guldberg, 1995) and superior competitive abilities relative to the brown morph (Takabayashi, 1994). Despite these correlations, the exact identity and function of this pigment remain elusive.

In this study, the pink pigment from the tissues of *P. damicornis* was isolated and characterized and its function was explored. Pigment complexes were also isolated from the pink morphs of the related pocilloporids *Stylophora pistillata* and *Seriatopora hystrix*, and the blue regions of colonies of the acroporids *Acropora formosa* and *A. digitifera*. The color in all five cases is associated with a similar protein complex, which in the case of *P. damicornis* does not appear to function as a photoprotectant, UV-screening agent, or fluorescent coupling pigment.

Materials and Methods

This is the first study to purify the pigment complex associated with the pink color of *Pocillopora damicornis*. To simplify the description of this compound in the following text, the compound is referred to hereafter as "pocilloporin." Similar compounds from other species (*i.e.*, with the same molecular weight, subunit size, or both) will be referred to as "pocilloporin-like" compounds.

Purification of pocilloporin and pocilloporin-like compounds

Corals (*Pocillopora damicornis, Seriatopora hystrix, Stylophora pistillata, Acropora digitifera*, and *A. formosa*) were collected at a depth of 2 m from One Tree Island lagoon near the One Tree Island Research Station (University of Sydney) at the southern end of the Great Barrier Reef, Australia, in May 1994. Pigments were extracted by immersing coral branches in 0.06 *M* KH_2PO_4, 0.06 *M* K_2HPO_4 pH 6.65 (phosphate buffer = "raw extract"; Takabayashi and Hoegh-Guldberg, 1995) for 24 h at 4°C. Raw extracts were concentrated and partially purified by centrifugation (Centrifuge 17RS, Heraeus Sepatech) through a Centricon 30 (Amicon, molecular weight cutoff = 30 kD, time and speed determined by volume and Centricon specifications). Wavelengths of maximum absorbance (λ_{max}) and protein concentrations were determined spectrophotometrically (Pharmacia Ultrospec III and Autofill III; Whitaker and Granum, 1980).

Gel filtration. Pocilloporin (from *P. damicornis*, λ_{max} = 560 nm) and pocilloporin-like proteins (from *Seriatopora hystrix, Stylophora pistillata*, and the *Acropora* species, λ_{max} = 560–590 nm) were further purified by gel filtration on a Superose FPLC column (Pharmacia, 12 HR 10/30). The sample was eluted from the column with phosphate buffer pH 6.65 at a flow rate of 0.5 ml min^{-1} and the absorbance of the protein was monitored using a multi-wavelength detector (Model 490E; Millipore-Waters, Australia). The major peaks were collected and analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) to determine subunit molecular weights. Collected fractions were rerun through the HPLC to determine purity (symmetry and overlay of 280-nm peak and 560-nm peak) and the extinction coefficient for pocilloporin at 560 nm (see below).

SDS-PAGE gel electrophoresis. Polyacrylamide gel electrophoresis (15% running gel) in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed using a modification of the method described by Laemmli (1970). In general, β-mercaptoethanol (5%) was added to all samples and the samples were boiled for 5 min prior to loading on the gel. However, to study the effects of boiling and reducing conditions on the mobility of sample subunits during electrophoresis, boiled and unboiled samples were run in the presence and absence of β-mercaptoethanol, sodium dodecyl sulfate (SDS), or both. In these experiments, and others involving SDS-PAGE, protein subunits were revealed by Coomassie blue staining (Righetti *et al.*, 1990). All gels used Biorad low molecular weight standards.

Properties of isolated compounds

Relationship between pocilloporin and co-eluting protein. To investigate whether pocilloporin was a protein, gel filtration was done on extracts of regions of two colonies of *P. damicornis* that varied in the intensity of pink color. These extracts were used to investigate the relationship between absorbance at 560 nm and co-eluting protein. The relationship between protein abundance (280-nm absorbance, Dawson *et al.*, 1986) and pigment (560-nm absorbance) was measured by relating the area of a defined 280 slice (Fig. 4A) to that of the corresponding 560 slice (Maxima 820 software; Millipore-Waters, Australia). All chromatograms were collected on the same day to minimize the effects of changes in column performance. The same start and end time points were used for delimiting chromatogram slices.

Measurement of extinction coefficient at 560 nm for pocilloporin. The extinction coefficient of pocilloporin, ϵ_{560}, was measured using Beer's law (Nobel, 1983), where the path length of the detector (Model 490E; Millipore-Waters, Australia) was 1 cm, and where the values for A_{560}

and the molar concentration (M) of pocilloporin were derived from 280-nm and 560-nm chromatograms of purified pocilloporin (Fig. 1A). The molar concentration of pocilloporin was calculated in the following manner. The amount of pocilloporin (micrograms) was calculated by converting the area of a very slim "slice" of the 280-nm chromatogram (Area A, Fig. 1A) to protein concentration, using a relationship previously determined between the total area under a 280-nm chromatogram and known amounts of protein from several different colonies of *Pocillopora damicornis* injected through the column (protein in micrograms = 8.89 × area + 0.10, $r^2 = 0.95$). The volume of each slice was calculated by multiplying the x-axis (time elapsed, Fig. 1A) of the slice by the flow rate (0.5 ml · min^{-1}). The resulting concentration of pocilloporin (grams per liter) was then converted into the molar concentration (M) of pocilloporin by using the native molecular weight of pocilloporin (= 54 kD, see Results). This method was used to determine the extinction coefficient because it required only relatively small amounts of protein and thus could be applied to only the purest of fractions (determined by observation of the symmetrical overlay of the 560-nm and 280-nm chromatograms).

To verify the validity of the above method, the extinction coefficient for pocilloporin at 560 nm was also determined using a more conventional technique employing two methods of measuring protein concentration (Bradford, 1976; Whitaker and Granum, 1980). Five aliquots of raw extract that had been molecular weight filtered (using Centricons) were injected into the gel filtration column, and the pocilloporin fractions collected. The collected fractions were pooled and concentrated, and the absorbances were measured at 235, 280, and 560-nm with a spectrophotometer (Pharmacia Ultraspec III).

Ion content of pocilloporin. Many chromophores include a chelated metal ion (Fox, 1979). To determine whether pocilloporin has a constituent metal ion, the ion content of pure pocilloporin was investigated. Pocilloporin was purified by gel filtration as described above. About 20 μg of protein (10 μl) was placed in 70% nitric acid (AristaR, BHD Chemicals) for 4 h at 95°C, and diluted to 3 ml of 0.7% HNO_3 with Milli-Q distilled water. Ion content was then determined by inductively coupled plasma mass spectroscopy (ICP-MS; Elan 5000, Perkin Elmer) using the total quant peak-hopping option. The blank contained 10 μl of phosphate buffer eluant from HPLC heated in 70% nitric acid (AristaR, BHD Chemicals) and diluted to 3 ml of 0.7% HNO_3 with Milli-Q distilled water.

Thermal lability of pigment compound. The pocilloporin fraction was collected as it eluted from the column. Fractions were concentrated by centrifugation through a Centricon 30. The concentrated sample was aliquoted into 25-μl proportions in 0.6-ml Eppendorf microcentrifuge tubes. Samples were then held in a water bath at temperatures of 40°, 60°, and 100°C for 10 min before being injected onto the column. A control (RT, Fig. 5) sample was injected without incubation in the water bath. Peak area was determined at both 560 nm and 280 nm, and the ratio of 560 nm to 280 nm was determined (Maxima 820 software; Millipore-Waters, Australia). In a separate experiment, pocilloporin was introduced into the (60°C) cell of a spectrophotometer, heated to 60°C, and maintained at this temperature for 10 min, during which it was scanned from 200 nm to 900 nm once every 80 s to determine changes in absorbance profile with heating (Pharmacia Ultraspec III).

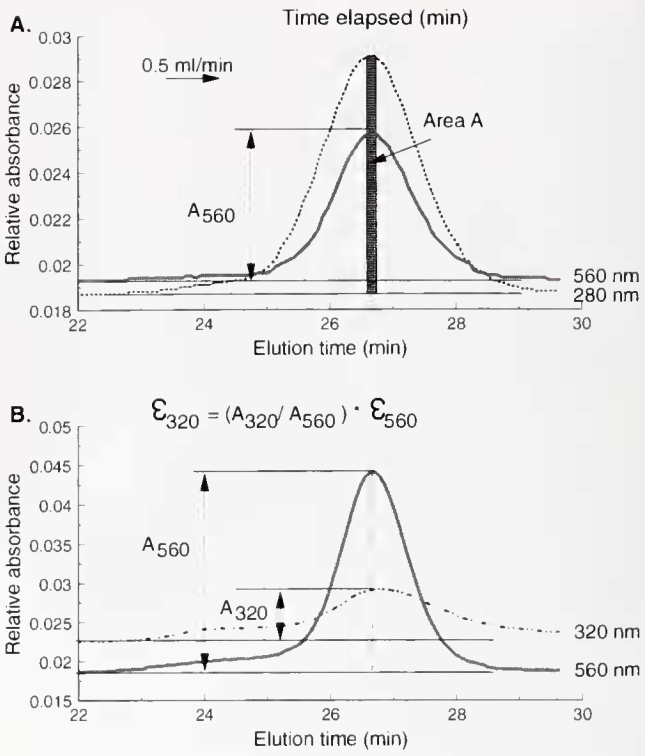

Figure 1. (A) Example of purified pocilloporin showing symmetrical overlay of 560 nm and 280 nm chromatograms and labeled parameters used in the calculation of the extinction coefficient at 560 nm. (B) Calculation of the extinction coefficient for pocilloporin at 320 nm from the extinction coefficient at 560 nm.

Solubility of pocilloporin and pocilloporin-like compounds. Ethanol, acetone, ether, and chloroform were added to separate phosphate buffer extracts (50% v/v each) of the coral tissues. Solubility was checked by looking for a precipitate in samples (1.5 ml) after vortexing and allowing samples to settle.

Effect of denaturing agents on pigment. Five percent SDS was added to phosphate buffer extract of pigment from *P. damicornis*. The raw extract, and the extract after the addition of the denaturing agent, were scanned from 200 nm to 700 nm (Pharmacia Ultraspec III) to measure any bathochromic shifts (λ_{max} shifts). Phosphate buffer extracts from all five coral species were acidified with

H_3PO_4 and neutralized with NaOH. Samples were vortexed and spun for 1 min in an Eppendorf microcentrifuge E, prior to spectrophotometric and visual examination.

The extinction coefficient and the contribution of pocilloporin to the total absorbance of Pocillopora damicornis *at 320 nm.* The extinction coefficient of pocilloporin at 320 nm (ϵ_{320}) was calculated by multiplying the ϵ_{560} for pocilloporin by the ratio of the absorbance at 320 nm to that at 560 nm for four purified samples (Fig. 1B). The greatest possible contribution of pocilloporin to the total UV absorbance (320 nm) was measured in the following manner for five pink colonies of *Pocillopora damicornis.* The total absorbance at 320 nm was measured for raw extracts after they were filtered through glass fiber filters (Millipore) to remove suspended material (no color remained on the filter). The filtered raw extracts contain both mycosporine amino acids (Matthews, 1993) and pocilloporin. The portion of the total 320-nm absorbance due to pocilloporin was calculated from the absorbance at 560 nm of the raw filtered extract multiplied by the ratio of ϵ_{320} to ϵ_{560} (only the pocilloporin fraction of raw extracts absorbs at 560 nm, Fig. 2A). This value was then expressed as a percentage of the total absorbance at 320 nm.

Fluorescent emission measurement. The fluorescent emission (between 390 and 750 nm) of pocilloporin was measured for excitation at 390 and 560 nm (both absorption maxima) with a luminescence spectrometer (LS50B, Perkin Elmer). Phosphate buffer extracts of pink (0.3 mg/ml pocilloporin by Beer's law from ϵ_{560} with A390 = 0.505) and brown (0.1 mg/ml pocilloporin with A390 = 0.519) *P. damicornis* branches, and a partially purified extract of pink branches (retentate after centrifugation through a Centricon 30; 3.7 mg/ml pocilloporin) were used for these measurements. If pocilloporin is to act as an accessory photosynthetic pigment *via* fluorescence, then the concentrations of pocilloporin used in these measurements should yield measurable fluorescence (*cf.* 0.15 μg/ml chlorophyll with A436 = 0.006 provided a detectable fluorescent response for excitement at 436 nm).

Results

Purification of pocilloporin from Pocillopora damicornis

Phosphate buffer extracts of pink and brown morphs of *P. damicornis* had similar complex 280-nm chromatograms (Fig. 2A, D). The key features of these chromatograms were as follows: (i) A peak that also absorbs at 320 nm (data not shown) eluting at 37 min (Fig. 2A, D; MAA), which corresponds to a molecular mass of about 1.3 kD. The 1.3-kD peak contained mycosporine-like amino acids (MAAs) as shown by C18 reverse phase HPLC (data not shown). Previously, MAAs have been shown to elute though gel filtration columns at times that correspond to compounds of about 1.3 kD despite having an actual molecular weight of 0.4 kD (Matthews, 1993). (ii) A void volume peak (v_o) that contains proteins of molecular mass greater than 2000 kD and absorbs slightly at 560 nm (Fig. 2A–F). (iii) A group of overlapping peaks occurring between 20 and 30 min that absorb at 280 nm (Fig. 2A, B, D, E). In this region, the pink morph of *P. damicornis* has a major 560-nm absorbing peak at 26.8 min with a small peak on the front shoulder (Fig. 2C).

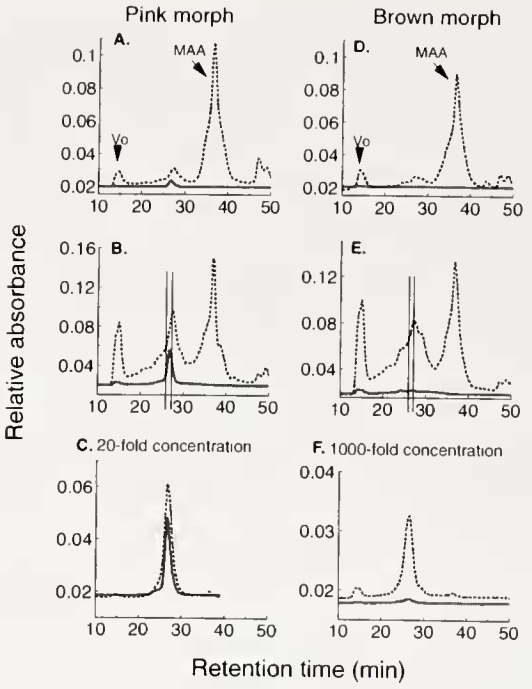

Figure 2. Comparison of the pink and brown morphs of *Pocillopora damicornis,* showing the purification steps for the 560-nm chromatogram peak (pocilloporin). (A and D) Phosphate buffer pigment extracts: V_o indicates void volume; MAA indicates UV-absorbing peak. (B and E) Retentate after centrifugation through a Centricon 30: ‖ indicates 560-nm fraction collected. (C and F) Chromatograms of fractions collected from B and E, respectively. Note concentration difference between C and F.

Centrifugation of the phosphate extract through a Centricon 30 reduced the relative quantity of UV-absorbing compound (*cf.* Fig. 2B, E with Fig. 2A, D). Further purification of the pink fraction revealed that it co-eluted with a 280-nm absorbing fraction, presumably protein with a native molecular weight of about 54 kD (Fig. 2C). Fractions collected at the same elution time (*i.e.,* corresponding to 54 kD proteins) from the brown morphs of *P. damicornis* did not show significant absorbance at 560 nm (Fig. 2F), even when concentrated 1000-fold (as opposed to 20-fold for the pink morph) from the previous step (Fig. 2E, B). Concentrating the purified fractions from the brown morph by 1000-fold resulted in an amplification of contaminating proteins (see end of next paragraph).

SDS-PAGE of pocilloporin fraction (Fig. 2C) from a pink morph of *P. damicornis* showed one major band with a molecular weight of 28 kD (Fig. 3; lanes 4, 5). Other bands that are present were due to contamination inasmuch as their appearance is dependant on the start and end time of fraction collection (*cf.* narrow collection periods shown in Fig. 3; lanes 4, 5, with broader collection period in Fig. 8 *P. damicornis;* lane 5). The presence or absence of β-mercaptoethanol or boiling or SDS did not affect the mobility of this 28-kD band (data not shown), suggesting that disulfide bonds are not involved in the linking of the subunits in the native protein. SDS-PAGE of the same gel filtration fraction taken from a brown *P. damicornis* morph (Fig. 2F) showed a very faint band at 28 kD amongst a smear of other bands with a wide molecular weight range (Fig. 3; lanes 1, 2).

Properties of pocilloporin and related compounds

Relationship between pocilloporin and co-eluting protein. The association between the absorbance at 560 nm and co-eluted protein was investigated to strengthen the conclusion that pocilloporin was a protein present in pigmented coral morphs but absent in unpigmented morphs. The 280-nm chromatograms of brown fragments of *P. damicornis* had a concave shape in the region of the maximal absorbance at 560 nm (26.8 min, Fig. 4A). The 280-nm chromatograms of pink fragments of *P. damicornis* had a convex shape in this region (Fig. 4A). The shape of these curves suggests the absence of a protein in the brown morph that is present in the pink morph of *P. damicornis.* That this protein is bound to or part of the pigmented compound is supported by the strong positive correlation

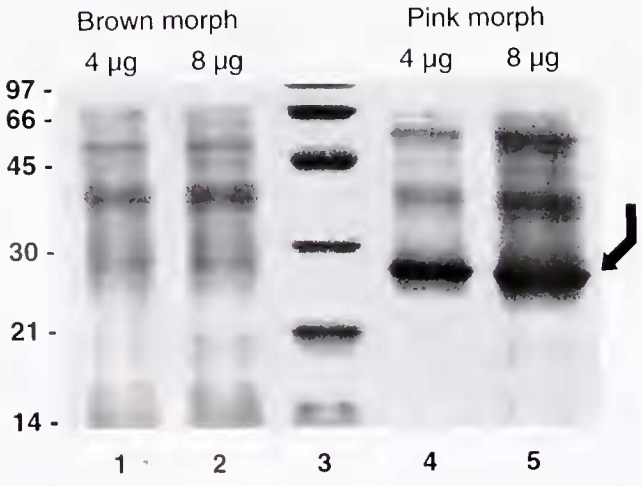

Figure 3. Fifteen percent SDS-PAGE of 560-nm fraction from pink and brown morphs of *Pocillopora damicornis:* Lane 1, 2, brown morph (4 and 8 µg, respectively); lane 3, Biorad low MW standards; lane 4, 5, pink morph (4 and 8 µg, respectively). The band corresponding to a 28-kD subunit is indicated by an arrow.

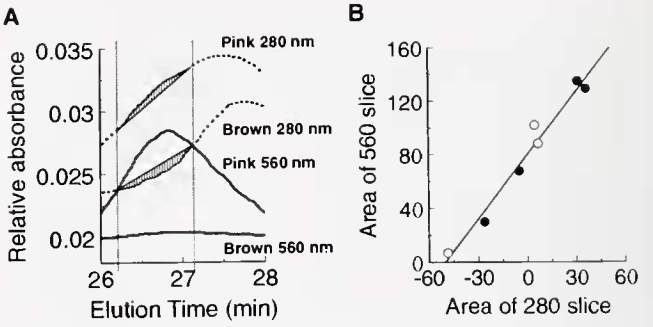

Figure 4. Relationship between absorbance at 560 nm and 280 nm. (A) Chromatograms of phosphate buffer extracts from pink and brown branches of *Pocillopora damicornis;* shaded slices show area used in 280 determination. (B) Linear relationship between area of 560 slice and area of 280 slice: $r^2 = 0.97$; (●) colony 1; (○) colony 2.

between the 560-nm absorption and 280-nm absorption (Fig. 4B; linear relation with $r^2 = 0.97$).

Extinction coefficient. The extinction coefficient (ϵ_{560}) of pocilloporin was determined directly from three independent chromatograms of purified pocilloporin (*e.g.,* Fig. 1A) and was 34059 ± 1635 cm^{-1} M^{-1} (mean ± SEM). The extinction coefficient measured using a spectrophotometer applied to purified protein from five HPLC runs was 31950 cm^{-1} M^{-1} (using the method of Whitaker and Granum, 1980, to measure protein) and 32900 cm^{-1} M^{-1} (using the method of Bradford, 1976, to measure protein). The three methods resulted in extinction coefficients for pocilloporin that were not statistically different ($P > 0.05$), hence verifying the validity of the first method.

Metal ion analysis. The association of metal ions with pocilloporin was investigated using ICP-MS. Total-quant analysis of the ion content of pocilloporin samples revealed no ions occurring at significantly greater levels than background; therefore, pocilloporin does not have an accompanying metal ion in its structure.

Thermal lability of pocilloporin. Chromatograms at 280 nm and 560 nm of pocilloporin from a broad collection around 560 nm are asymmetrical (Fig. 5A), demonstrating that the fraction (in this case) was contaminated with proteins other than pocilloporin. No changes occured to pocilloporin when it was heated to 40°C for 10 min. When heated to 60°C, 280-nm and 560-nm absorbance decreased, as did the relative amount of 560-nm absorbance to 280-nm absorbance. The decrease in the ratio of absorbance at 560 nm to 280 nm may be due to the fact that the contaminants are more thermally stable than pocilloporin. At 100°C, there was a further decrease in both 280- and 560-nm absorbances (Fig. 5A). Spectrophotometric scans of pocilloporin reveal that no bathochromic shifts accompanied the loss of 280-nm and 560-nm absorbance (Fig. 5B; note, baseline shift for sample maintained at 60°C for 10 min). Heating raw extract to 100°C changes the solution to pale yellow. This correlated

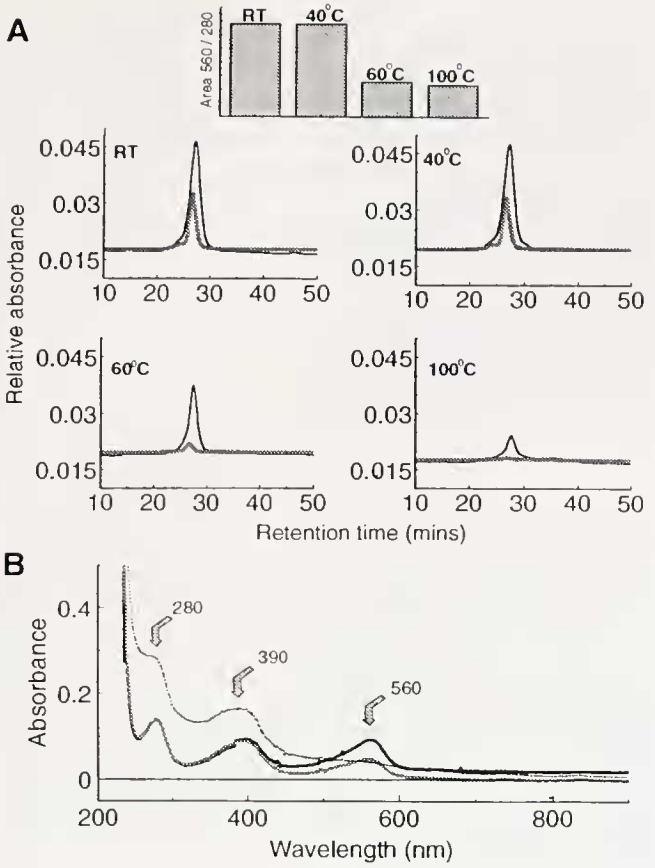

Figure 5. Thermal lability of 560-nm fraction from *Pocillopora damicornis*: RT = control chromatogram; 40°C = chromatogram after heating sample to 40°C for 10 min; 60°C = chromatogram after heating sample to 60°C for 10 min; 100°C = chromatogram after heating sample to 100°C for 10 min. (—) 280-nm, (xxxx) 560-nm chromatograms. Bar chart, showing ratio of 560-nm peak area to 280-nm peak area at each temperature. (B) Wavelength scan of pocilloporin at different temperatures: (—) cell at 25°C, (xxxx) cell at 60°C, (- - -) cell at 60°C after 10 min. Arrows indicate approximate position of λ_{max} in each case.

well with the observation that the 390-nm peak was relatively unaffected by temperature.

Solubility in solvents and the effects of denaturing agents on pigment compounds. For pink *P. damicornis*, 5% SDS gave a white pellet and a colorless supernatant with no absorbance between 360 and 700 nm. Ethanol and acetone gave a pinky-purple precipitate and a colorless supernatant. The supernatant had no peak absorbance between 360 and 700 nm, the precipitate redissolved in phosphate buffer, and the solution had peak absorbances (λ_{max}) at 560 and approximately 385 nm. Colored extracts from all coral species examined were insoluble in ethanol, acetone, ether, and chloroform. In all coral species examined, acidification (pH 4.8) or alkalization (pH 11.2) of phosphate buffer extract altered the color of solutions of pocilloporin to pale orange, with further acidification or alkalization turning solutions yellow and giving rise to a yellow precipitate.

The importance of UV-absorbance by pocilloporin. The ϵ_{320} for pocilloporin was calculated to be 14889 $\pm$ 64 cm^{-1} M^{-1} (mean $\pm$ SEM; n = 4 chromatograms). The proportion (percent) of the total absorbance at 320 nm in raw extracts that was due to pocilloporin was 1.60 $\pm$ 0.49% (mean $\pm$ SEM; n = 5).

Fluorescent emission measurement. Fluorescence (390–750 nm) was not emitted for excitation at 560 nm, from either unpurified extracts of brown and pink *P. damicornis* colonies or partially purified extracts of pocilloporin. Some fluorescence was observed for samples excited at 390 nm. Brown fragments of *P. damicornis* fluoresced at 450 nm and 480 nm. Pink fragments fluoresced only at 450 mm. The intensity of the fluorescence was, however, unrelated to the concentration of pocilloporin in the sample (pink and brown extracts with the same 390-nm absorbance fluoresced with the same intensity at 450 nm). Fluorescence, as a result of 390-nm excitation, was therefore due to compounds other than pocilloporin in raw and partially purified extracts of *P. damicornis*.

Comparison of pocilloporin-like compounds: native and subunit molecular weights within and between families

Stylophora pistillata, *Seriatopora hystrix*, *Acropora digitifera*, and *A. formosa* have 280-nm chromatograms

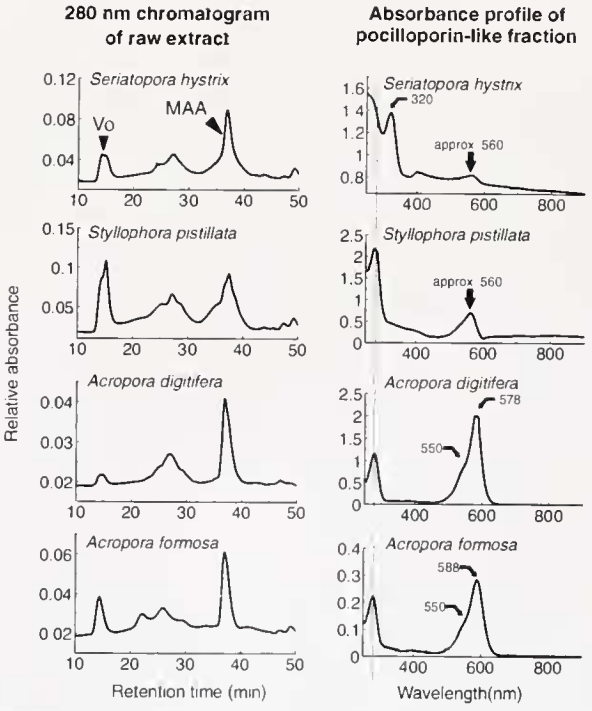

Figure 6. Chromatograms of raw phosphate buffer extracts from four different coral species at 280 nm; V_o, void volume; MAA, mycosporine-like amino acids. λ_{max} determinations of isolated pocilloporin-like compounds. Arrows indicate approximate λ_{max}. Vertical dotted line indicates position of 280-nm absorbance.

similar to those of *Pocillopora damicornis*. All five species have a peak related to the presence of mycosporine-like amino acids that elutes at about 37 min (MAA, Fig. 6), a group of peaks that elute between 20 and 30 min, and a void volume peak that elutes at approximately 14 min (V_o, Fig. 6). *S. hystrix* and *S. pistillata* had λ_{max} about equal to 560 nm (Fig. 6). *A. formosa* had λ_{max} about equal to 578 nm and a shoulder at about 550 nm (Fig. 6). In the blue *Acropora* species, *A. formosa*, there is a shift in the native molecular weight (MW) of the pigment from 54 kD (native MW of *P. damicornis*, *S. hystrix*, *S. pistillata*, and *Acropora digitifera*) to 82.6 kD (*cf.* peak position relative to dotted line, Fig. 7). However, in all coral species examined, SDS-PAGE of corresponding gel filtration fractions showed a single band with a subunit molecular weight of about 28 kD (Fig. 8, data not shown for *A. digitifera*). The front shoulder fractions, which absorb minimally at λ = max (560–590) nm and variably at 400 nm (Fig. 7), show a more complex banding pattern that contains both the 28-kD subunit and a 40-kD subunit amongst a smear of other bands (Fig. 8: *S. pistillata*, lane 1; *P. damicornis*, lane 1; *S. hystrix*, lane 5). Gel filtration chromatograms suggest that compounds absorbing at about 400 nm are more closely associated with a peak whose approximate molecular weight is 112 kD than with the pocilloporin or pocilloporin-like compound peak (Fig. 7).

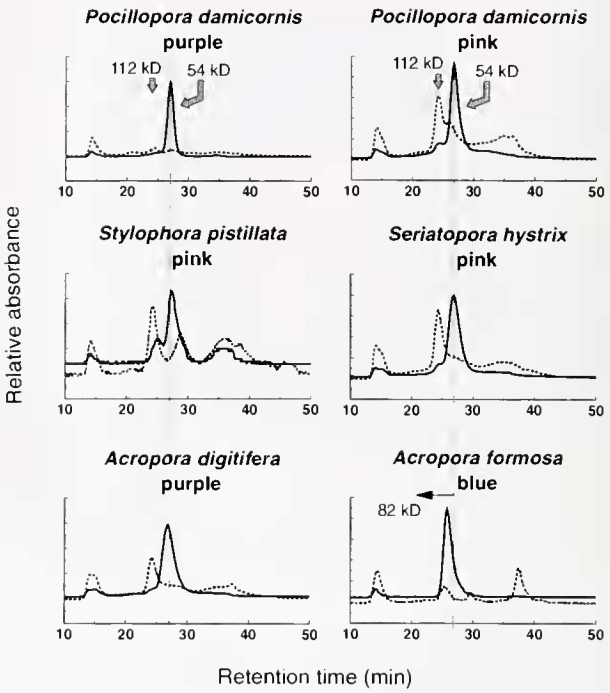

Figure 7. 400-nm (- - -) and 560-nm (—) chromatograms from Centricon 10 (Amicon) centrifuged phosphate buffer extracts from *Pocillopora damicornis*, *Stylophora pistillata*, *Seriatopora hystrix*, *Acropora digitifera*, and *A. formosa*. Dotted line shows approximate position of 54-kD eluting protein.

Discussion

The colors that typify many members of the animal kingdom have a variety of roles that range from crypsis to courtship (McFall-Ngai, 1990; Dawkins and Guilford, 1993). Although the role of color has been explored extensively in some groups (*e.g.*, insects: Endler, 1981; birds: Owen, 1980; fish: Neal, 1993), an understanding of the function of color in others is lacking. Reef-building corals fall into the latter category. This study is the first attempt to isolate and biochemically characterize the compounds responsible for the pink and blue colors of two prominent families of reef-building corals (Pocilloporidae, Acroporidae). Once the protein dimer from *Pocillopora damicornis* (pocilloporin) had been isolated and characterized, its function was explored, and several functions suggested by early studies were solidly rejected.

Biochemical structure of pocilloporin

SDS-polyacrylamide gel electrophoresis and gel filtration of pocilloporin and pocilloporin-like compounds suggest that the pigments from *Pocillopora damicornis*, *Seriatopora hystrix*, *Stylophora pistillata*, and *Acropora digitifera* are protein dimers with native molecular weights of about 54 kD and subunits of 28 kD (Figs. 1–3, 7, 8). The pigment from the blue regions of *A. formosa* is a trimer with a native molecular weight of about 82.6 kD and subunits of 28 kD (Figs. 7, 8). The subunits in the case of *P. damicornis* are not linked by disulfide bonds in the native protein.

All evidence supports the existence of a strong stoichiometric link between the protein and chromophore in pocilloporin. The nature of that link can be explained in several ways. That is, the absorbance at 560 nm could be due to (i) a non-protein compound that is coincidentally eluting with a same-sized protein without any chemical bonding between them; (ii) a chromophore that is non-covalently bonded with a protein to form a chromophore-protein complex, and (iii) a chromophore covalently bonded to the protein.

The first option can be eliminated by the strong correlation between the absorption at 560 nm and the concentration of co-eluting 54-kD protein from colonies differing in the expression of the pink pigment (Fig. 4). In other words, the more of the particular protein there is, the greater the absorbance at 560 nm. Furthermore, a 28-kD subunit existed in significant amounts only in the fractions with a high absorbance at 560 nm and not in fractions that had no absorbance at 560 nm (*e.g.*, extracts of brown colonies, Fig. 3). The second option, that the chromophore is non-covalently linked to the protein, is questionable on the grounds that the chromophore is not easily separated from the protein.

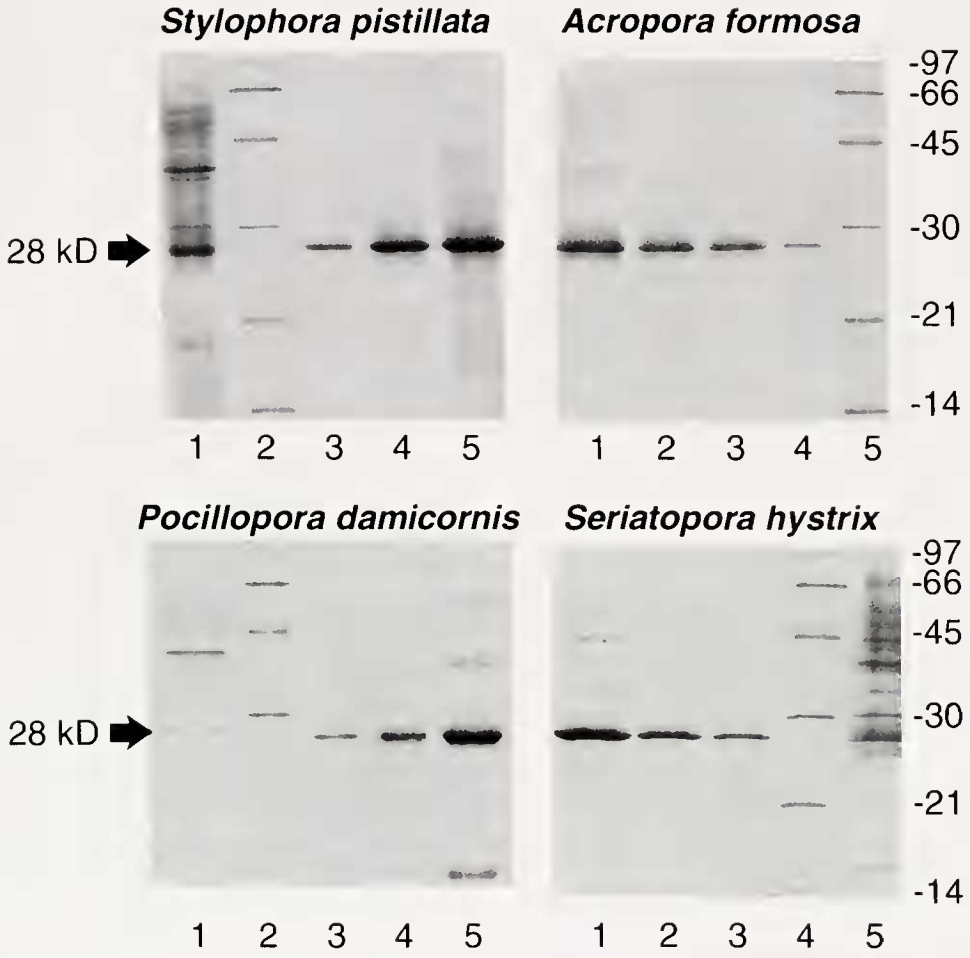

Figure 8. Fifteen percent SDS-PAGE of isolated pigment (pink or blue) fractions from different coral species. *Stylophora pistillata:* lane 1, front shoulder of 560-nm peak (8 μg); lane 2, Standards; lanes 3–5, main 560-nm fraction with 2, 4, 8 μg, respectively. *Acropora formosa:* lanes 1–4 main 580-nm fraction with 8, 6, 4, 2 μg, respectively. *Pocillopora damicornis:* lane 1, front shoulder of 560 peak (4 μg); lane 2, Standards; lanes 3–5, main 560-nm fraction with 2, 4, 6 μg, respectively. *Seriatopora hystrix:* lanes 1–3, main 560-nm fraction with 6, 4, 2 μg, respectively; lane 3, Standards; lane 5, front shoulder of 560-nm peak (8 μg).

Treatments such as 5% SDS, heat, and relatively nonpolar solvents such as acetone liberate carotenoids and other chromophores from their conjugate proteins, resulting in bathochromic shifts in the absorbance of the pigment whilst the protein either denatures (5% SDS, heat) or precipitates out of nonpolar solvents (Cheeseman *et al.*, 1967; Fox, 1979; Milicua *et al.*, 1985; Garate *et al.*, 1986; Zagalsky *et al.*, 1989; Zagalsky *et al.*, 1991). No such reaction was found with pocilloporin or the pocilloporin-like compounds of this study. Treatments with these agents simply resulted in precipitation of the pigment complex from solution.

Pocilloporin-like compounds from the Pocilloporidae and Acroporidae

Several other biochemical properties of pocilloporin and pocilloporin-like compounds were identified in this study. The compounds were hydrophilic, in agreement with the work of Kawaguti (1944) and Shibata (1969), who both extracted similar coral pigments in water. It is curious, however, that Shibata (1969) did not find an absorption peak at 560 nm in the water extracts of *Pocillopora* sp.; the main absorbance peak found in that study was at 480 nm. However, Shibata did not classify the species of *Pocillopora* he used, and his description of the morph as red rather than pink suggests that it may have been a differently pigmented form than the ones used in the present study. Shibata (1969) also described pigments from *Acropora* sp. as having absorbance peaks at 560 nm and 590 nm. Our results for two species of *Acropora* are similar: *A. digitifera* had a λ_{max} at 578 nm and a shoulder at 550 nm. Similarly, *A. formosa* had a λ_{max} at 588 nm and a shoulder at 550 nm (Fig. 6).

The present study did not determine the biochemical pigment group to which the proteins pocilloporin and pocilloporin-like compounds belong; however, some options seem unlikely. No metal ions were found associated with pocilloporin, thus eliminating it from the group of metal-bearing biochromes such as the colored copper proteins and metal-chelated tetrapyrroles (Fox, 1979). The absence of metal ions cannot be attributed to inadequate sample size. Assuming 1 metal ion per protein molecule, we would expect readings in the range of parts per million for the amount of protein introduced into the ICP-MS. This amount is well above the detection capabilities of the ICP-MS, which can measure ion concentrations down to the range of parts per billion (Henshaw *et al.*, 1989). The absorbance of pocilloporin at 560 nm is significantly reduced by short (10-min) exposures to 60°C without any accompanying bathochromic shift (Fig. 5). This extreme thermal sensitivity suggests that this absorbance is not due to more thermally stable compounds such as carotenoids (*e.g.*, those that retain absorbance even when heated at temperatures up to 120°C for 1 h, Lee *et al.*, 1987, 1990). Pocilloporin, therefore, does not appear to involve a carotenoid component and is probably not a carotenoprotein.

Why be pigmented? Possible roles of pocilloporin

Investigation of the biochemical characteristics of pocilloporin did not reveal its function. However, several possible functions are firmly rejected and a narrow range of possibilities remains. The following hypotheses about the function of pocilloporin are rejected.

Pocilloporin as a photoprotectant. In his seminal study of coral color, Kawaguti (1944) proposed that pigments may shade zooxanthellae from excessive sunlight that might otherwise lead to photobleaching of constituent photosynthetic pigments. The results of the present study are counter to this idea. The absorption spectrum of pocilloporin suggested that it did not protect against photobleaching of the major photosynthetic pigments because its absorbance spectrum does not coincide with those of chlorophyll *a* and *c* (Nobel, 1983; Kirk, 1994) and is, if anything, curiously complementary to these compounds. Thus, a role for pocilloporin as a photoprotectant for the photosynthetic pigments of the zooxanthellae is unlikely.

Pocilloporin as an accessory photosynthetic pigment. If pocilloporin were an accessory photosynthetic pigment, the energy absorbed by it would have to be transferred to the photosystems in the zooxanthellae to be used for photosynthesis. This transfer could occur in two ways. The first way is by the direct energy transfer (resonance energy transfer). In this case, light energy absorbed by one molecule is passed to the reaction centers in the photosystems through a chain of energy transfers between closely adjacent molecules (Nobel, 1983). This could happen only if pocilloporin were in proximity to the photosystems. However, most (if not all) of the pocilloporin is associated with the coral tissues (Takabayashi and Hoegh-Guldberg, 1995) and not with the zooxanthellae. Consequently, the direct transfer of excitation energy by resonance transfer from pocilloporin to photosystems is impossible. A second method of energy transfer is through fluorescent coupling. In this case, pocilloporin might absorb light at non-photosynthetic wavelengths and re-fluoresce them at wavelengths suitable for absorption by the primary photosynthetic pigments (Kawaguti, 1944; Schlichter *et al.*, 1988). This type of transfer has been proposed for pigments associated with the animal tissues of the deep-water coral *Leptoseris fragilis*, which transforms short-wavelength, non-photosynthetically active radiation into longer wavelength, photosynthetically active radiation, which is reabsorbed by the photosynthetic pigments of the symbiotic dinoflagellates (Schlichter and Fricke, 1991). This indirect means of energy transfer is, however, unlikely for pocilloporin, which is not fluorescent for excitation at either of its λ_{max}.

Pocilloporin as a UV-screening pigment. The last possibility is that pocilloporin might have UV-absorbing capabilities in addition to its ability to absorb visible light. This is also unlikely because the absorbance of pocilloporin within the UV region of light was minimal (1.6% of the total absorbance of raw extracts at 320 nm). Compounds such as the mycosporine-like amino acids that are abundant in corals are far more potent UV-B-screening agents (Dunlap and Chalker, 1986), a fact that is clear from the comparison of the ϵ_{320} of pocilloporin (14,889 $cm^{-1}\ M^{-1}$) with the ϵ_{320} of a typical mycosporine-like amino acid (*e.g.*, ϵ_{320} of palythine = 36,200 $cm^{-1}\ M^{-1}$, Dunlap and Chalker, 1986). These MAAs, especially palythine (λ_{max} = 320 nm), are probably responsible for more than 95% of the absorption by the coral at 320 nm (Dunlap and Chalker, 1986; Dunlap *et al.*, 1988).

The role of pocilloporin remains elusive. Pocilloporin may act as an agent that enhances the abilities of the pigmented morph to resist fouling or predation or to compete successfully (Lang, 1971, 1973; Sheppard, 1979, 1982). It is interesting to note that Rinkevich and Loya (1983) reported the purple morph of *Stylophora pistillata* from the Red Sea to be competitively superior to the yellow morph of the same species, even when they were not touching. Takabayashi (1994) found a similar trend in the competitive abilities of the pink morph of *Pocillopora damicornis*. In this case, the pink morph won significantly more of the contests between pink and brown colonies in grafting experiments, and a distinct pink band was often observed in the tissue of pink colonies at the contact sites between nonsimilar colonies. Pocilloporin, therefore, might function in the coral's immunological and chemical defense systems.

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